

Characterization and Purification of a Phytotoxin Produced by *Fusarium solani*, the Causal Agent of Soybean Sudden Death Syndrome

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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ABSTRACT

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A phytotoxic polypeptide identified in culture filtrates of *Fusarium solani*, the causal agent of soybean sudden death syndrome, was heat unstable, negatively charged, absorbed by 10% charcoal, and destroyed by proteinase K. The toxicity of the culture filtrates and fractions obtained during purification was bioassayed by measuring browning of soybean

calli. Purification of the phytotoxin was achieved by Sephadex G-50 gel filtration chromatography followed by ion exchange chromatography on a DE-52 column. The purified protein migrated as a single band on sodium dodecyl sulfate-polyacrylamide gels with an estimated molecular weight of 17,000. The sequence of the N-terminal 15 amino acids was determined and indicated that a peptide was present. Samples containing this single protein caused browning of soybean calli, necrosis on detached soybean cotyledons and leaves, and yellowing, curling, and drying of attached soybean cotyledons and leaves.

Sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Merr.) is caused by the soilborne fungus, *Fusarium solani* (Mart.) Appel & emend. Syd. & Hans. (18,19). Symptoms of SDS include root rot, crown necrosis, vascular discoloration of roots and stems, interveinal chlorosis and necrosis of leaves, and premature defoliation and pod abortion (9). In a previous study, culture filtrates of *F. solani* SDS-causing isolates were phytotoxic to soybean callus, cotyledons, germinating seeds, and plants (14). Jin et al. (10) showed, using five soybean cultivars, that susceptibility to the fungus was positively correlated with calli sensitivity to the fungal filtrates. *F. solani* isolates from alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris*), lupine (*Lupinus albus*), pea (*Pisum sativa*), and potato (*Solanum tuberosum*) did not cause SDS symptoms on inoculated soybean plants, and their culture filtrates had significantly lower toxicity to soybean calli than did SDS-causing isolates (10). In other studies, monorden, a low molecular weight phytotoxin also from cultures of *F. solani* SDS-causing isolates, inhibited shoot and root growth and caused leaf and stem necrosis of soybean plants (1).

Numerous other phytotoxins also have been isolated from cultures of *Fusarium* spp. For example, two phytotoxic isomeric compounds, marticin and isomarticin, were isolated from *F. solani* f. sp. *pisi* and other *Fusarium* spp. (12). Anhydrofusarubin, fusaru-

bin, and javanicin were identified in culture extracts of a *F. solani* isolate from citrus roots and severely inhibited root growth of radish seedlings (2). Fusaric acid (5-*n*-butylpicolinic acid), produced by several *Fusarium* spp. associated with wilt of banana, cotton, pea, tomato, and other plants (12), affected membrane permeability by inhibiting proton extrusion and increasing leakage of potassium ions and other electrolytes and inhibited respiration of *Egria densa* (15). The pathogenicity of *F. oxysporum* f. sp. *niveum* was positively correlated with the fusaric acid content of diseased plants (7). Lycoramasmin, a polyamino acid produced by *F. oxysporum* f. *lycopersici*, was found in wilted tomato plants (25). Enniatins (cyclodepsipeptides) were produced by several *Fusarium* spp. (8). When enniatin was added as a solution to germinating wheat seeds, decreased seedling growth was directly related to increased enniatin concentrations (5). All of the phytotoxins mentioned above have low molecular weights (<1,000) (8,14).

Although no proteinaceous toxins (>10 kDa) have previously been described from *Fusarium* spp., there are several reports of involvement of higher molecular weight polypeptides in disease development from other fungi. Several host-selective toxic proteins produced by *Pyrenophora tritici-repentis*, the causal organism of tan spot of wheat, have been purified and characterized (3,22,24). In tomato leaf mold, extracellular proteins (14-kDa ECP1 and 17-kDa ECP2) produced by *Fulvia fulva* (synonym *Cladosporium fulvum*) were purified and immunolocalized in diseased tomato plants (11,27). Necrosis-inducing peptides of 3.8, 6.8, and 9.2 kDa were produced by *Rhynchosporium secalis*, the causal agent of barley leaf scald (26).

In this paper, the characterization and purification of a 17-kDa proteinaceous phytotoxin from an SDS-causing isolate of *F. solani* is described.

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MATERIAL AND METHODS

Culture filtrates. *F. solani* isolate 269 (provided by L. E. Gray) was grown in a modified Septoria medium (modified Septoria medium (MSMJ) (21) containing full-strength micronutrients, instead of one-fourth strength. Cultures were grown in 2-liter flasks, each containing 400 ml of culture medium, or 500-ml flasks, each containing 100 ml of culture medium. The liquid medium was inoculated with a conidial suspension to give 1×10^4 conidia per ml of medium and incubated without shaking in the dark at 23°C for 12 days. Cultures (12-day-old) were filtered through Whatman No. 1 filter paper (Whatman, Clifton, NJ) and a 0.22- μ m Millipore membrane (Millipore Corp., Bedford, MA).

Calli bioassay. Calli of soybean cvs. Asgrow A3427 and Spencer were used to measure phytotoxicity of fungal culture filtrates (10). Culture filtrates, adjusted to pH 5.8 with 5 N HCl, were filtered through sterile 0.22- μ m Millipore membranes and added to autoclaved calli medium (Murashige and Skoog medium [16] amended with 0.5 mg of 6-benzylaminopurine per liter and 5 mg of α -naphthaleneacetic acid [NAA] per liter) at a ratio of 5:100 (vol/vol) before the medium solidified. For each test, each of three plates (100 \times 15 or 60 \times 15 mm) contained either 16 or 9 pieces of calli per plate, respectively. These were incubated at 28°C under a 16-h photoperiod (60 μ E m⁻² s⁻¹). Calli browning either was visually evaluated as plus (browning) or minus (no browning) or spectrophotometrically as the absorbance of acetone extracts of calli at 330 nm after growing 5 days on filtrate- or MSM-amended media. Extracts were prepared by grinding 0.1 g of calli in 4 ml of 80% acetone with a glass tissue grinder. The solution was centrifuged at 1,000 \times g for 4 min, and the absorbance of the supernatant was measured at 330 nm (10).

Phytotoxin production. The effect of media on toxin production was evaluated using eight media. Five of the eight media (100 ml each) were placed in 500-ml flasks: CDB = Czapek Dox broth, FL = a defined medium for growing *F. solani* citrus isolates (1), PDB = potato dextrose broth, V8 juice, and MSM. In another experiment, three additional media were prepared from plant-tissue extracts and compared to MSM. Plant tissue from either soybean cotyledons (2 g), leaves, or stems was ground with distilled water and filtered separately through Whatman No. 1 filter paper. Each of the filtrates from plant tissue was adjusted to 100 ml with distilled water, and 1 g of sucrose was added (pH not adjusted) prior to autoclaving at 121°C for 20 min. Media from the two experiments were inoculated with *F. solani* isolate 269, and culture filtrates were obtained as described above. The phytotoxicity of culture filtrates and media without fungus was tested by calli bioassay as described above. The phytotoxicities were compared as follows: (extract absorbance of calli treated with culture filtrate produced on test medium/extract absorbance of calli treated with culture filtrate produced on MSM) \times 100.

To determine the effect of pH on phytotoxin production, isolate 269 was grown on MSM with the pH adjusted with 1 N HCl or 1 N NaOH to 3.0, 4.5, 5.8, 7.5, or 9.0 and incubated without shaking in the dark at 23°C for 12 days. Fresh weight of mycelium was recorded, and phytotoxin production was evaluated with calli extracts. The data were analyzed by analysis of variance, and treatment means were compared by Fisher's protected least significant difference test ($P = 0.05$).

To determine the relationship between fungal growth and phytotoxin production, isolate 269 was grown in eight 250-ml flasks, each containing 50 ml of MSM. Two flasks were harvested weekly for four consecutive weeks to collect culture filtrates. Fungal growth was measured as fresh weight of mycelia. The pH of the culture filtrate was measured, and the phytotoxin production was assessed by calli bioassay. In a similar experiment, culture filtrates were collected from two flasks at 3-day intervals for 27 days. Both experiments were repeated.

Phytotoxin characterization: heat treatment. Culture filtrate

(5 ml) obtained from 12-day-old cultures of isolate 269 grown on MSM was autoclaved at 121°C for 20 min and incorporated into 100 ml of calli medium. Phytotoxicity of culture filtrates was recorded as plus (calli browning) or minus (no calli browning). Culture filtrates (5 ml) also were incubated at 4, 28, 37, 50, 75, and 100°C for 30 min and tested on calli. Experiments were repeated once.

Ethyl acetate extraction. Culture filtrates were extracted with ethyl acetate (1:1, vol/vol) three times at pH 1, 3, 5.8, 7, 8.5, 11, and 12. Both water and ethyl acetate phases were incorporated into calli medium at 2:100 and 4:100 (vol/vol). A 1.3-ml portion from the water phase of the 4:100 (vol/vol) ratio was filter-sterilized and poured on a 7-cm-diameter sterilized filter-paper disk, air-dried under sterile conditions, and placed on a plate with 33 ml of calli medium (4%). A 1.3-ml portion from the ethyl acetate phase was air-dried and dissolved in 1.3 ml of filter-sterilized acetone. The acetone solution was poured on a sterilized filter-paper disk, air-dried under sterile conditions, and placed on a plate with calli medium. Acetone alone was applied to a sterilized filter-paper disk and placed on a plate with calli medium as control. Calli of cv. Asgrow A3427 or Spencer were placed on the filter paper on the calli medium and incubated as described above. Phytotoxicity of culture filtrate was recorded as plus (calli browning) or minus (no calli browning). Both the water- and ethyl acetate-phase experiments were replicated three times.

Estimation of molecular weight. Culture filtrates were dialyzed against distilled water with dialysis tubing (Spectrum Medical Industries, Inc., Houston) with a molecular weight cutoff of either 6,000 to 8,000 or 12,000 to 14,000 for 48 h. Dialysis tubing was pretreated at 100°C for 15 to 20 min in a solution of 1% NaHCO₃ and 0.1% Na₂EDTA (6). Filtrate remaining inside the tubing was tested for phytotoxicity on calli. Fungal filtrates also were centrifuged with microconcentrator with a molecular weight cutoff of 30,000 (Microcon-30, Amicon, Inc., Beverly, MA). Filtrates in both the upper and lower levels were tested for phytotoxicity on calli. Fungal filtrates were concentrated five times with a Speed Vac (Appropriate Technical Resources, Inc., Laurel, MD), and a 0.5-ml sample was applied to an high-pressure liquid chromatography (HPLC) semipreparative column (Alltech macrosphere GPC [Alltech Ass., Inc., Deerfield, IL]: pore size 60 Å, particle size 7 μ m length \times inside diameter [i.d.] 300 \times 7.5 mm) connected in a series to another semipreparative column (Perkin-Elmer PE TSK G2000sw [Norwalk, CT]: pore size 130 Å, particle size 10 μ m, length \times i.d. 300 \times 7.5 mm). Both columns were preequilibrated with 50 mM KH₂PO₄, 150 mM Na₂SO₄ buffer, pH 6.0. The sample was eluted with the same buffer at a flow rate of 0.3 ml min⁻¹, and 0.9-ml fractions were collected. Phytotoxicities of fractions were tested on cv. Asgrow A3427 calli. Molecular weight was estimated by retention time with standard molecular weight markers: bovine serum albumin (66,000), carbonic anhydrase (29,000), chymotrypsinogen A (23,200), lysozyme (14,400), and cytochrome C (12,300).

Charge. Ion exchanger resins were used to characterize culture filtrates. Dry CM-cellulose (20 g) was pretreated and preequilibrated with 0.03 M Na acetate, pH 4.5 (6), and was added to 1.5 liters of fungal filtrate and stirred for 2 h at room temperature. The CM-cellulose was removed by vacuum filtration through Whatman No. 1 filter paper. The filtrate, adjusted to pH 5.8 with 5 N HCl, was tested for phytotoxicity on cv. Spencer calli.

DEAE-cellulose (20 g) was pretreated and preequilibrated with 0.5 M Tris-Cl at pH 7.8 (6) and was added to 1.5 liters of fungal filtrates and stirred for 2 h at room temperature. The DEAE-cellulose was removed by vacuum filtration through Whatman No. 1 filter paper. The filtrate, adjusted to pH 5.8, was tested for phytotoxicity on Spencer calli.

Charcoal. Fungal filtrates were mixed with charcoal (1 or 10 mg ml⁻¹). The charcoal was removed by vacuum filtration through a 0.22- μ m Millipore membrane, and the filtrates were tested for phytotoxicity on cvs. Asgrow A3427 and Spencer calli.

Proteinase treatment. Proteinase K (Sigma Chemical Company, St. Louis) was added to 20 ml of culture filtrate (0.05 mg ml⁻¹) and incubated in a water bath at 37°C for 2 h. Digestion was terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) (Sigma, 0.5 mM). Culture filtrate also was added to either peptidase (Sigma, 4 mg ml⁻¹) or pepsin (Sigma, 10 mg ml⁻¹) and incubated at 37°C for 2 h. The reaction solution, adjusted to pH 5.8, was filtered through a sterile 0.22- μ m Millipore HA filter (Bedford, MA) and applied to calli medium as previously described. Controls included PMSF (0.1 or 0.5 mM), culture filtrate added to PMSF (0.1, 0.5, or 1 mM), and three kinds of enzyme solution alone. Cv. Spencer calli were transferred to the medium; 5 days later calli browning was recorded. The experiment was repeated once.

Ammonium sulfate precipitation. Ammonium sulfate was added to fungal filtrates at concentrations of 30 to 100% (wt/vol) in 10% increments, and the mixtures were kept on ice for 15 min. The solutions were centrifuged at 10,000 \times g for 20 min at 0°C. The precipitates were dissolved in distilled water, and both the supernatant and precipitate portions were dialyzed (12 to 14 kDa cutoff) against water for 24 h and tested for phytotoxicity on cv. Spencer calli.

Isolate specificity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of fungal filtrates from isolates causing SDS of soybean and isolates not causing SDS of soybean (10) was performed on 15% polyacrylamide slab gels with a Bio-Rad Mini-protein II electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) to view the difference in extracellular proteins produced by these isolates. Fungal culture filtrates were mixed with sample buffer at 1:1 (vol/vol) and boiled for 4 min. Each minigel lane was loaded with 25 μ l of sample. Electrophoresis of two slab gels was run under constant 50 mA current for about 1 h. The gels were silver-stained (4), and the protein molecular weights were calculated by comparison with standards (6).

Purification. Fungal culture filtrates (1 liter) were concentrated with a spiral cartridge membrane S1Y10 (Amicon Division, W. R. Grace & Co., Beverly, MA) to 150 ml (10 kDa cutoff), and further concentrated by rotary evaporation at room temperature to less than 20 ml. The concentrated filtrates were centrifuged, and the supernatant was applied to a Sephadex G-50 (Sigma) column (2.6 \times 100 cm) preequilibrated with 50 mM Tris-Cl, 100 mM NaCl, and pH 7.5 elution buffer at 4°C. The column was eluted with buffer at a flow rate of 0.8 ml min⁻¹, and 8-ml fractions were collected. The phytotoxic fractions were combined and applied to a DE-52 (Whatman LabSales, Hillsboro, OR) column (30 cm length, 2.5 cm diameter) preequilibrated with 50 mM Tris-Cl and pH 7.5 buffer. The column was eluted with a 0 to 1 N NaCl gradient in 50 mM Tris-Cl (pH 7.5). The eluates of both columns were monitored at 280 nm, and the phytotoxic fractions, as detected by calli bioassay or SDS-PAGE, were combined. The pooled fractions were dialyzed against distilled water and dried with a Speed Vac.

Native PAGE of the sample was performed as described for SDS-PAGE, except that the sodium dodecyl sulfate and 2-mercaptoethanol were omitted. The 17-kDa band was excised from the native polyacrylamide gel and placed on calli medium. Cv. Asgrow A3427 calli were placed on top of and to the side of the gel and incubated for 2 to 3 days. The partially purified samples (50 μ l) containing a single band were placed on pipette tip-wounded detached cotyledons, with a toxin concentration of from 0.01 to 1.5 μ g μ l⁻¹. The toxin (100 μ l) also was applied on pipette tip-wounded detached leaves (0.5 to 1.5 μ g μ l⁻¹), and 0.5 to 15 ng μ l⁻¹ also was applied to seedlings of cvs. Asgrow A3427 and Ripley with roots excised for 2 to 3 days.

N-terminal amino acid sequencing. SDS-PAGE of the partially purified phytotoxin was performed on a 10 \times 20-cm slab gel under a constant 30 mA current for 6 h. The amount of protein was estimated by staining the gel with Coomassie brilliant blue R-250 (0.25%, wt/vol) in a solution containing methanol (45%, vol/vol)

and glacial acetic acid (10%, vol/vol) for 2 h and destaining in a solution of methanol (40%, vol/vol) and glacial acetic acid (10%, vol/vol) overnight. Proteins separated by SDS-PAGE were electroblotted onto polyvinylidene difluoride (PVDF) transfer membranes. The protein band (17 kDa) (0.1 to 10 μ g) was excised from the membrane sheet and used for amino terminal sequencing by the Biotechnology Center, University of Illinois at Urbana-Champaign, Urbana.

RESULTS

Phytotoxin production. Culture filtrates from all five media in which *F. solani* was grown, except FL, caused calli browning. Filtrate from MSM was most toxic to calli, whereas filtrate from FL was least toxic (2% of the MSM toxicity). The remaining media caused intermediate browning, as measured by their percent absorbance compared to that of MSM. PDB was at the lower end of the range at 43%, and CDB was highest at 78% absorbance compared to MSM, as measured by absorbance at 330 nm of calli acetone extracts (Fig. 1). The final fresh weight of *F. solani* grown on FL also was the lowest. An initial MSM pH of 3.0 to 9.0 did not significantly ($P = 0.05$) affect the fungal growth or phytotoxicity of the culture filtrates. The toxicity of the culture filtrates, as measured by the absorbance of calli extracts (browning), peaked when the culture filtrates were sampled at 9 days, which was 3 days after maximal fungal growth (Fig. 1).

Phytotoxin characterization. To characterize the toxic compound(s), the culture filtrate was treated in various ways, and the toxicity was measured by calli bioassay. Autoclaved culture filtrates did not cause calli browning, and toxicity decreased when the culture filtrate was exposed to temperatures above 50°C for 30 min (Fig. 2). The 100°C treatment for 30 min reduced toxicity by approximately 75%.

The ethyl acetate phase of the fungal filtrates extracted at different pHs did not cause browning, whereas calli did turn brown on medium amended with the water phase. Filtrates retained by the dialysis tubing (up to 12 to 14 kDa cutoff) caused calli browning that was not visually distinguishable from that caused by non-dialyzed culture filtrates. The filtrates in the upper level of Ultra-free CM filter (30 kDa cutoff) did not cause calli browning, whereas the lower molecular weight compounds (<30,000) caused calli browning. When the culture filtrate was analyzed by HPLC, the fraction with a retention time of 54 min contained the toxic activity. The molecular weight of the component in this fraction was from 14,000 to 23,000. The toxic constituent was removed by

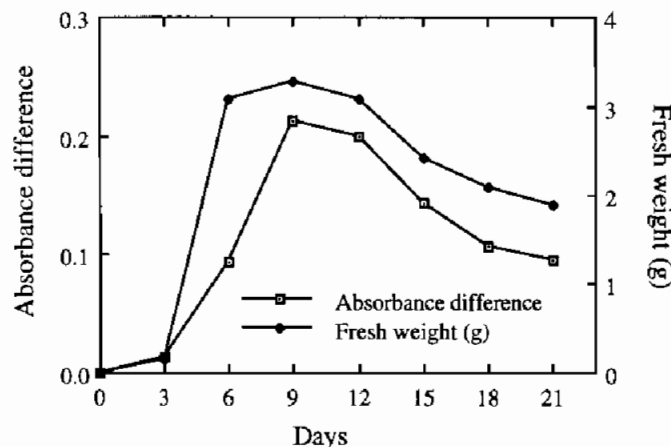


Fig. 1. Fresh weight of *Fusarium solani* grown in a modified Septoria medium and absorbance (at 330 nm) of extracts of soybean calli grown on calli medium amended with culture filtrate (5:100, vol/vol) collected from fungal liquid cultures at 3-day intervals up to 21 days. Absorbance difference = A_{330} (culture filtrate amended) - A_{330} (nonamended).

DEAE-cellulose but not by CM-cellulose treatment. Because the phytotoxin was adsorbed by the anion exchanger, DEAE-cellulose, this indicated that it was negatively charged. Crude fungal filtrates treated with 10%, but not 1%, charcoal did not cause calli browning. Culture filtrates treated with 0.05 mg of proteinase K per ml were not toxic to cv. Spencer calli, whereas those treated with 4 mg of peptidase or 10 mg of pepsin per ml caused calli browning. Controls such as enzyme alone and 0.5 mM PMSF, which was added to inhibit proteinase K, showed no toxicity, whereas 0.5 mM PMSF plus the culture filtrate was toxic. The toxic constituent was not precipitated by 30 to 50% ammonium sulfate but was partially precipitated with 60% or more. The precipitate, but not the supernatant, from culture filtrates treated with 100% ammonium sulfate was toxic to calli. All of these results indicate that the toxic compound was a protein. When culture filtrates of the different *F. solani* isolates were run on SDS-PAGE, only the *F. solani* SDS-causing isolates contained a polypeptide of 17 kDa that was not observed in the filtrate of the other isolates (Fig. 3).

Purification. The crude culture filtrate was brown, but gel filtration on a Sephadex G-50 column removed most of the colored material from the toxic fraction, and the toxicity was not associated with any peak of absorbance but was eluted consistently at one-half of the bed volume (5 h of elution) (Fig. 4). Chromatography on a DE-52 column showed the toxicity was associated with a major peak of absorbance that eluted at about 1 h (Fig. 5).

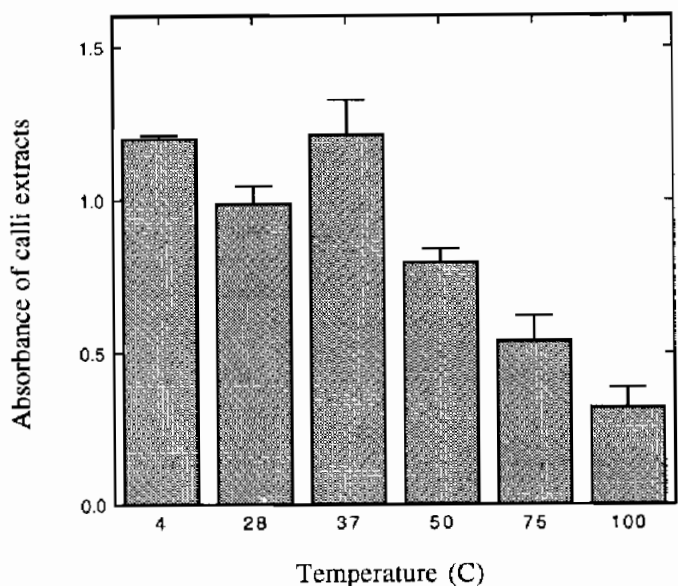


Fig. 2. Absorbance at 330 nm of soybean calli extracts grown for 5 days on media containing *Fusarium solani* culture filtrates that had been incubated, at the temperatures listed, for 30 min. Vertical bars indicate standard error.

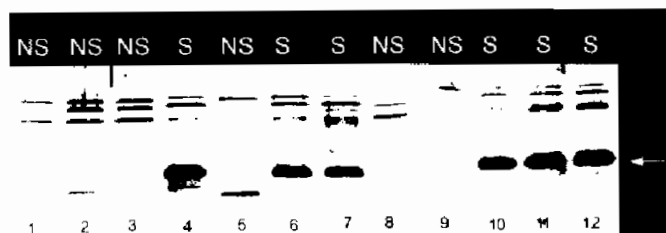


Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of culture filtrates of *Fusarium solani* isolates from: lane 1, potato; lanes 2 and 8, bean; lane 3, lupine; lanes 4, 6, 7, 10, 11, and 12, soybean; lane 5, alfalfa; and lane 9, pea grown on a modified Septoria medium for 12 days. The gel was silver-stained. The arrow indicates a band of about 17 kDa. NS = isolates not causing sudden death syndrome (SDS) on soybean; S = isolates causing SDS on soybean.

The phytotoxin purified by Sephadex G-50 and DE-52 column chromatography migrated on sodium dodecyl sulfate and native polyacrylamide gels as a single band. When the single protein band found on the native gel was excised and soybean calli were placed on or near it on solid medium, this section caused calli browning, whereas other gel bands were not toxic. The sample containing the 17-kDa protein band also caused necrosis on detached leaves (Fig. 6). Toxin concentrations as low as 2.5 ng μl^{-1} pro-

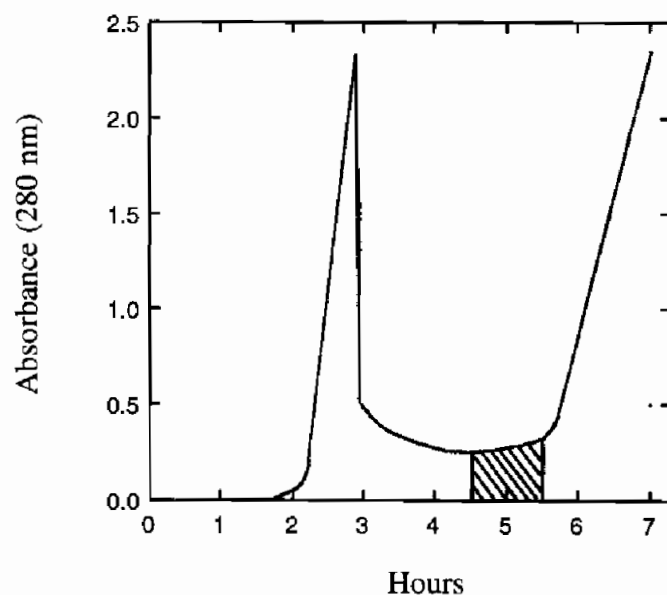


Fig. 4. Gel filtration on a Sephadex G-50 column of concentrated culture filtrates of *Fusarium solani*. The phytotoxic activity peaked at approximately 5 h of elution as measured by the calli bioassay.

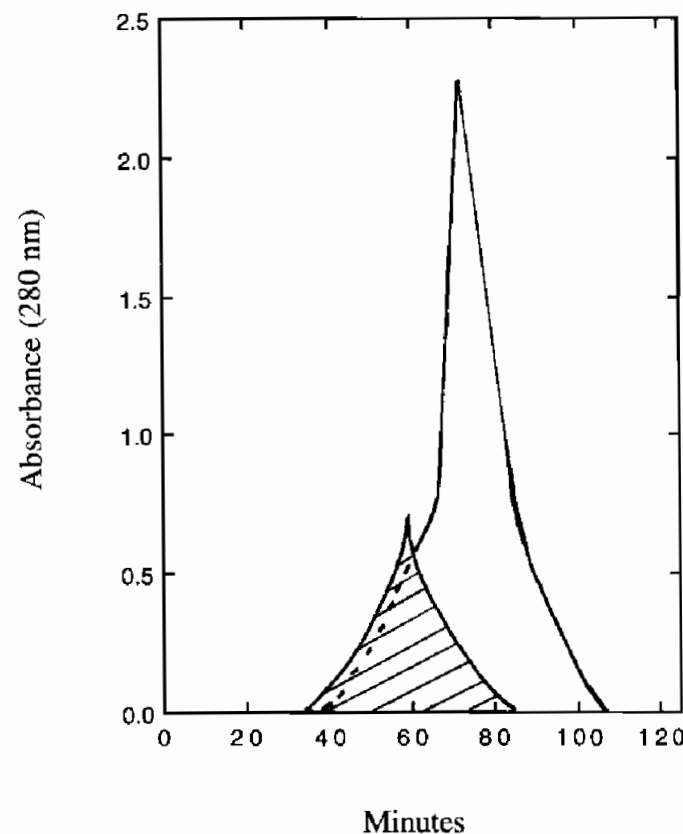


Fig. 5. Ion exchange chromatography on a DE-52 column of a pool of toxic fractions eluted from Sephadex G-50 column chromatography. The shaded area indicates toxic activity as measured by the calli bioassay.

duced a yellow discoloration on cotyledons, and $15 \text{ ng } \mu\text{l}^{-1}$ caused leaf curling and drying after a 2-day incubation period. When the purified protein band was presented for amino acid sequencing, the N-terminal was not blocked, so the sequence of the N-terminal 15 amino acids was Ala Thr Gln Phe Ser Tyr Thr Gly Ser (Glu) Thr Gly (Thr) Asp Gln.

DISCUSSION

We found previously (10) that the phytotoxicity of *F. solani* culture filtrates was greater on calli of soybean cultivars that were more susceptible to SDS, as shown by symptom development on inoculated plants. This calli bioassay was used to assess toxicity during the purification of a 17-kDa phytotoxic peptide from the culture filtrate. This peptide, which was present only in *F. solani* isolates that did not cause SDS, produced necrosis on soybean cotyledons and leaves. Thus, unlike most phytotoxins produced by *Fusarium* spp., this toxin is proteinaceous and has no similarity to monorden, a low molecular weight (364), ethyl acetate-extractable compound produced by *F. solani* SDS-causing isolates (1). Monorden, like the 17-kDa phytotoxic polypeptide identified in this study, was produced by soybean SDS-causing isolates but not by isolates that did not cause SDS (1). Neither toxin has been detected in plants expressing SDS symptoms. It is possible that both toxins are involved in the symptomology of SDS, and it also is possible that these toxins are produced under different culture and environmental conditions. The development of antibodies against these toxins may permit their detection in diseased soybean plants, as has been done with naphthazarin, a toxin produced by *F. solani* in citrus (17), and with the Ptr necrosis toxin (13).

In our studies, the 17-kDa protein identified in culture filtrates of a *F. solani* SDS-causing isolate had some similarities to Ptr toxins (3,23,24). Due to the similar molecular weight of the toxins, the crude concentrated culture filtrates from both fungi showed similarities when Sephadex G-50 gel filtration chromatography was done, and the phytotoxicity was not associated with any absorbance peak but was eluted consistently at one-half of the bed volume. Ion exchange chromatography also showed similarities, including the fact that the phytotoxicity was associated with a major absorbance peak at 280 nm in both cases. However, the phytotoxicity of the Ptr toxin was heat stable, whereas the toxicity of the 17-kDa *F. solani* toxin was completely lost after 20 min at 121°C and decreased by 75% after 30 min at 100°C . ECP2, an extracellular protein produced by the fungal tomato pathogen *F. fulva* had

the same molecular weight as the toxin in this study and could be localized in planta (27).

There are several other reports that showed that phytotoxic peptides were related to necrotic symptoms, including a 13-kDa protein produced by *Ceratocytis ulmi*, the causal agent of Dutch elm disease (22), and the Ptr toxin, which induced necrosis only on susceptible wheat cultivars (23). A 3.2-kDa peptide produced by *F. fulva*, the product of an avirulence gene, was a race-specific elicitor of necrosis (20). The barley leaf scald causal organism, *R. secalis*, produced necrosis-inducing peptides of 3.8, 6.8 and 9.2 kDa (27).

Because the protein characterized in our study has not been detected in soybean plants with SDS symptoms, further work is being done to develop an antibody against the toxin and to determine if other toxin(s) play a role in SDS symptom development. We have observed that foliar symptoms on different plants from the same soybean cultivar can vary, from the typical interveinal chlorosis to small necrotic flecks, which may indicate that different toxins or partial detoxification occurs within the plant to produce different symptoms. In addition, temperature seems to influence foliar symptoms, which also may have some effect on the activity of the toxin. Additional research needs to be conducted to determine the mode of action of the toxin(s) involved in SDS symptomology.

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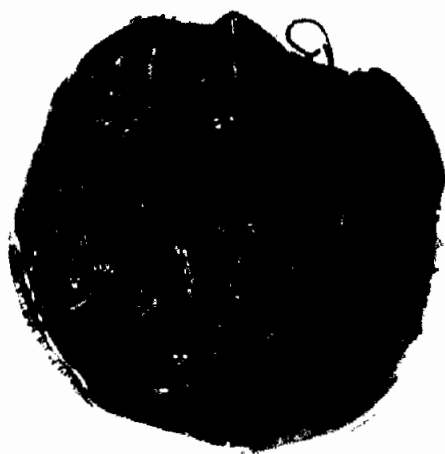


Fig. 6. Partially purified toxin ($50 \mu\text{l}$) of *Fusarium solani* on detached soybean cv. Ripley leaves after 2 days. Upper left leaflet with $0.1 \mu\text{g}$ of toxin per μl . Upper center leaflet with 0.05 (left) and 0.01 (right) of toxin per μl . Upper and lower right leaflets are controls. Lower left leaflet with $0.75 \mu\text{g}$ of toxin per μl . Lower center leaflet with 1.5 (left) and 0.2 (right) μg of toxin per μl .

duced a yellow discoloration on cotyledons, and $15 \text{ ng } \mu\text{l}^{-1}$ caused leaf curling and drying after a 2-day incubation period. When the purified protein band was presented for amino acid sequencing, the N-terminal was not blocked, so the sequence of the N-terminal 15 amino acids was Ala Thr Gln Phe Ser Tyr Thr Gly Ser (Glu) Thr Gly (Thr) Asp Gln.

DISCUSSION

We found previously (10) that the phytotoxicity of *F. solani* culture filtrates was greater on calli of soybean cultivars that were more susceptible to SDS, as shown by symptom development on inoculated plants. This calli bioassay was used to assess toxicity during the purification of a 17-kDa phytotoxic peptide from the culture filtrate. This peptide, which was present only in *F. solani* isolates that did not cause SDS, produced necrosis on soybean cotyledons and leaves. Thus, unlike most phytotoxins produced by *Fusarium* spp., this toxin is proteinaceous and has no similarity to monorden, a low molecular weight (364), ethyl acetate-extractable compound produced by *F. solani* SDS-causing isolates (1). Monorden, like the 17-kDa phytotoxic polypeptide identified in this study, was produced by soybean SDS-causing isolates but not by isolates that did not cause SDS (1). Neither toxin has been detected in plants expressing SDS symptoms. It is possible that both toxins are involved in the symptomology of SDS, and it also is possible that these toxins are produced under different culture and environmental conditions. The development of antibodies against these toxins may permit their detection in diseased soybean plants, as has been done with naphthazarin, a toxin produced by *F. solani* in citrus (17), and with the Ptr necrosis toxin (13).

In our studies, the 17-kDa protein identified in culture filtrates of a *F. solani* SDS-causing isolate had some similarities to Ptr toxins (3,23,24). Due to the similar molecular weight of the toxins, the crude concentrated culture filtrates from both fungi showed similarities when Sephadex G-50 gel filtration chromatography was done, and the phytotoxicity was not associated with any absorbance peak but was eluted consistently at one-half of the bed volume. Ion exchange chromatography also showed similarities, including the fact that the phytotoxicity was associated with a major absorbance peak at 280 nm in both cases. However, the phytotoxicity of the Ptr toxin was heat stable, whereas the toxicity of the 17-kDa *F. solani* toxin was completely lost after 20 min at 121°C and decreased by 75% after 30 min at 100°C . ECP2, an extracellular protein produced by the fungal tomato pathogen *F. fulva* had

the same molecular weight as the toxin in this study and could be localized in planta (27).

There are several other reports that showed that phytotoxic peptides were related to necrotic symptoms, including a 13-kDa protein produced by *Ceratocytis ulmi*, the causal agent of Dutch elm disease (22), and the Ptr toxin, which induced necrosis only on susceptible wheat cultivars (23). A 3.2-kDa peptide produced by *F. fulva*, the product of an avirulence gene, was a race-specific elicitor of necrosis (20). The barley leaf scald causal organism, *R. secalis*, produced necrosis-inducing peptides of 3.8, 6.8 and 9.2 kDa (27).

Because the protein characterized in our study has not been detected in soybean plants with SDS symptoms, further work is being done to develop an antibody against the toxin and to determine if other toxin(s) play a role in SDS symptom development. We have observed that foliar symptoms on different plants from the same soybean cultivar can vary, from the typical interveinal chlorosis to small necrotic flecks, which may indicate that different toxins or partial detoxification occurs within the plant to produce different symptoms. In addition, temperature seems to influence foliar symptoms, which also may have some effect on the activity of the toxin. Additional research needs to be conducted to determine the mode of action of the toxin(s) involved in SDS symptomology.

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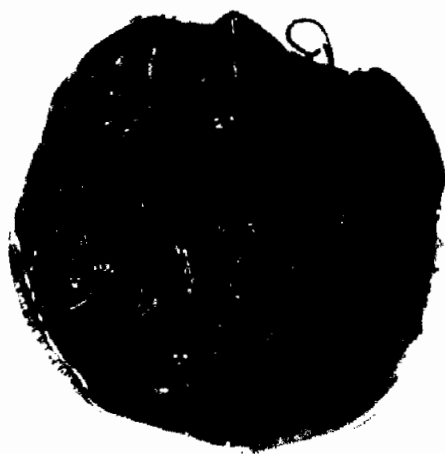


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